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RELATION OF THE ACTION OF CERTAIN BACTERIA TO THE RIPENING OF CHEESE OF THE CHEDDAR TYPE

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RELATION OF THE ACTION OF CERTAIN BACTERIA TO THE RIPENING OF CHEESE OF THE CHEDDAR TYPE[1]

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INTRODUCTION

The ripening of Cheddar as well as other varieties of cheese has been studied by a large number of investigators. The decomposition of the protein and the nitrogenous substances thereby produced have been quite thoroughly studied in Europe and America. These studies have involved both hard and soft cheeses. The nature of the nonnitrogenous substances formed during fermentation in cheese, such as fatty acids, alcohol, esters, and aldehydes, has received less attention, but there can be no doubt that they contribute to the aroma and also to the taste of the product. In their relation to flavor they are equally, if not more, important than the nitrogenous substances.

According to present views, the factors involved in the curing of Cheddar cheese are the pepsin contained in the rennet; the activating lactic acid formed from lactose fermentation; galactase, the proteolytic enzym of milk; other inherent enzyms of milk; and certain biological agents other than those simply concerned in the first lactose fermentation.

Investigations at the Wisconsin Agricultural Experiment Station and the New York (Geneva) Agricultural Experiment Station have shown that the inherent enzyms of milk and rennet fail to produce the typical Cheddar cheese flavor. This has led to a more extensive investigation of the biological factors of Cheddar cheese ripening.

In an earlier publication from the Wisconsin station (Suzuki, Hastings, and Hart, 1910)[2] both volatile acids and esters were separated and identified from curing Cheddar cheese, but no data concerning the factors operative in their origin were presented. In a later publication (Hastings, Evans, and Hart, 1912) work was reported that showed the presence and persistence in this type of cheese of three groups of organisms, the *Bacterium lactis acidi* group, the *B. casei* group,[3] and possibly a group of coccus forms.

[1] Work of the Department of Agriculture in cooperation with Wisconsin Agricultural Experiment Station.

[2] Bibliographic citations in parentheses refer to "Literature cited," p. 214–216.

[3] The organisms of the *Bacterium casei* group appear in the literature under a number of names, the most common being "lactic bacilli," "*Bacterium bulgaricus*" or "*Bacterium bulgaricum*," "*Bacterium casei*," and the "youghurt bacillus." The name "*Bacterium casei*" will be used in this article.

In a preliminary investigation of the nonnitrogenous constituents of Cheddar cheese (unpublished data) the very pronounced differences that were expected in the quantity and variety of volatile acids, esters, and alcohols in good and poor types of cheese were not found. But since there were certain differences which could be only of biological origin, it was believed essential to this problem that the substances formed by the specific groups of organisms normally present in cheese be more carefully studied. For this reason it was decided to extend the investigation to an examination of the substances produced by representatives of the groups that had been found to be present in cheese in such numbers that it was evident that they must be of importance in the ripening process. In this way it was hoped to find the groups of organisms to which might be assigned responsibility for the production of definite nonnitrogenous compounds that could be correlated with flavor production. The compounds particularly sought were the alcohols and esters and caproic and butyric acids. Formic, acetic, propionic, lactic, and succinic acids were also included in the list of substances to be isolated. To some extent the sources of these bodies were also studied. This paper is a progress report on this phase of our work.

Ferdinand Cohn (1875) was the first to connect the cheese-ripening process with the activity of bacteria. Duclaux (1894, p. 265–267) considered that the volatile fatty acids found in cheese arose from the action of the bacteria on casein and from the hydrolysis of the fat. He believed also that butyric acid was a source of other volatile acids, the butyric acid arising partly from fat decomposition and partly from decomposition of casein. Baier (1895), Von Klecki (1896), and Weigmann (1896, 1898) believed butyric-acid bacteria to be of importance in the ripening of cheese. Von Freudenreich (1897, 1902) attributed to the lactic-acid bacteria the principal rôle in the ripening process, especially in Emmenthaler cheese. Jensen (1904) in his work on Emmenthaler and other European cheeses has contributed much to the general subject of the chemistry and bacteriology of cheese ripening and in agreement with Von Freudenreich gives to the lactic-acid-producing organisms very great importance in the ripening process. Suzuki, Hastings, and Hart (1910) have investigated the source of the volatile acids and the forms of lactic acid found in American Cheddar cheese, studying in connection with these subjects the decomposition of lactose, lactates, fat, proteins, and glycerin.

The constituents of a fresh cheese mass which can be sources of the nonnitrogenous bodies under consideration are paracasein, fat, lactose, lactates, and citrates. From paracasein there arises gradually during the ripening process a series of nitrogenous compounds which have been fairly well investigated (Winterstein; Steinegger; Benecke and Schulze; Van Slyke and Hart, Apr., 1903, and July, 1903; Dox). At least three of these—namely, cadaverin, putrescin, and ammonia—are slightly

volatile and probably can influence the aroma of cheese. The other nitrogenous end-products undoubtedly are factors in the flavor production, and influence taste.

It is known that proteolysis gives rise also to volatile fatty acids, particularly butyric acid. In addition, milk fat, which is present to a large extent in the cheese, is a source of caproic and butyric acids through bacterial and enzymic action. The glycerin of the fat after hydrolysis by biological agencies is a source of acetic and propionic acids under the influence of further fermentation (Suzuki, Hastings, and Hart, 1910). That decomposition of fat occurs during cheese ripening, giving rise to caproic and butyric acids, has been shown by a number of workers. Duclaux (1894, p. 286) found that this occurred to quite an extent, giving rise to free volatile fatty acids. Weigmann and Backe (1898) point to the presence in ripe cheese of free nonvolatile acids, such as oleic, palmitic, and stearic, as an indication of fat decomposition in the cheese-ripening process. Kirsten (1898, p. 1), however, thought these higher acids could arise from paracasein and claimed that fat decomposition in ripening cheese is almost imperceptible. Jensen (1904, p. 319) has shown that very probably fat decomposition does take place with production of fatty acids during cheese ripening. The lactose fermentation produces, besides lactic acid, formic and propionic acids, and under certain conditions butyric and caproic acids also are formed (Suzuki, Hastings, and Hart, 1910). Calcium lactate, according to Fitz (1878, p. 51; 1879, p. 479; 1880, p. 1309; 1881, p. 1084), is a source of acetic and propionic acids, and under certain conditions also of caproic and valeric acids. Jensen (Von Freudenreich and Jensen, 1906, p. 320) and Troili-Petersson (1909, p. 333) have shown that the lactates in Emmenthaler cheese are fermented by organisms with the production of propionic and acetic acids and CO_2. Troili-Petersson has also shown that glycerin may be a source of propionic acid.

In an extended investigation (Evans, Hastings, and Hart, 1914) of the flora of American Cheddar cheese it has been shown that the organisms fall into four groups, the *Bacterium lactis acidi*, the *B. casei*, and two coccus groups.

The substances produced by the coccus groups form the principal theme of this paper. In addition, data are given on the substances formed from two representatives of the *Bacterium casei* group. In the following work pure cultures of several of the coccus forms known to occur in American Cheddar cheese were inoculated into flasks containing 300 c. c. of sterile separated milk and kept at a temperature of 35° C. for at least two months before being examined. No alkali whatever was added to the milk. The high-acid-producing organisms (*B. casei* group) were also inoculated into flasks of milk similarly prepared and incubated. Each culture was put up in duplicate flasks. The methods of analysis used were those described by Suzuki, Hastings, and Hart (1910). All

flasks subjected to analysis were examined to ascertain their freedom from growth of other organisms.

It has been observed that active lactic acid is the main form of this acid in fresh cheese curd, but that it rapidly changes to the racemic variety. In addition to the foregoing studies on substances formed by bacteria, this paper also includes some work done on the agencies which cause these changes in the form of lactic acid present in cheese and which take place during the earlier period of cheese ripening.

In the preceding article (Evans, Hastings, and Hart) the presence of coccus forms in normal Cheddar cheese is demonstrated. It is shown that nonliquefying cocci which ferment lactose in milk cultures are always present, in percentage of the total bacterial content ranging upward to 50. The cocci are distinguished from the *Bacterium lactis acidi* group by their morphology and by the extent of reduction of litmus in milk cultures. In cultures of the *B. lactis acidi* group the cells are in pairs, and some or all of the cells are elongated; there is always a characteristic reduction of litmus. The cocci include those cultures in which the cells are spherical. The complete reduction of litmus beneath the surface layer, characteristic of the *B. lactis acidi* group, does not take place.

A classification of the cocci occurring in this type of cheese is made. They are divided into two groups on the basis of morphology: Streptococci and micrococci. Those occurring in pairs are included with the streptococci, together with those which form chains of varying lengths. The microccocci are the Coccaceæ which divide in two planes; consequently the cells appear in pairs, fours, or bunches. Most of the cultures of this group produce a heavy growth upon agar slant, which is often of some shade of yellow. A further differentiation of the groups into varieties is made on the basis of fermentation of the following test substances: Lactose, salicin, sucrose, glycerin, and mannit. This classification of the cocci is given in Table I. The substances produced by representatives of several of these varieties have been analyzed, and the data are presented in Tables II to X.

TABLE I.—*Differentiation of the coccus groups into varieties*

Group.	Variety.	Production of acid in—				
		Lactose.	Salicin.	Sucrose.	Glycerin.	Mannit.
Streptococcus	a	−	−	−	−	−
	b	+	−	+	+	−
	c	+	+	+	+	−
	d	+	+	+	+	+
Micrococcus	b	+	+	−	−	−
	c	+	+	+	+	−
	d	+	+	−	−	+

ANALYSIS OF THE DECOMPOSITION PRODUCTS OF STERILE MILK

In Table II are given the quantities of the various substances found in 300 c. c. of the sterile milk after incubation for four months.

TABLE II.—*Decomposition products found in 300 c. c. of sterile milk*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity.		
	Flask 1.	Flask 2.	Average.
Total volatile acids	11. 763	12. 567	12. 164
Formic acid	5. 748	5. 348	5. 548
Acetic acid	5. 566	6. 756	6. 160
Propionic acid	. 000	. 000	. 000
Butyric acid	. 000	. 000	. 000
Caproic acid	. 449	. 463	. 456
Acids from alcohols	. 954	. 650	. 802
Acids from esters	. 685	. 600	. 640
Succinic acid	. 000	. 000	. 000
Total lactic acid	. 000	. 000	. 000
Racemic lactic acid	....	....	....
Active lactic acid	....	....	....

The occurrence of formic and acetic acid in the controls may be due to the decomposition of lactose in the process of sterilization. Formic acid, at least, has been observed in milk heated for some time at high temperature (Cazeneuve and Haddon).

The cultures of Streptococcus b_1 (Table III) were 8 weeks old when analyzed. Little digestion of the medium was apparent. The medium had a clean, sweet, fruity, or nutty taste and odor, was gray white in color, and somewhat slimy. This organism was present in the cheese to the extent of 10,000,000,000 per gram when isolated. The cheese was 77 days old when examined. It had a very mild Cheddar flavor, which developed late in the curing, and it afterwards developed a good sharp flavor.

TABLE III.—*Substances formed by the action of Streptococcus b_1*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids	52. 689	54. 930	12. 164	40. 525	42. 766
Formic acid	. 000	. 000	5. 548	−5. 548	−5. 548
Acetic acid	49. 920	50. 810	6. 160	43. 760	44. 650
Propionic acid	2. 575	4. 120	. 000	2. 575	4. 120
Butyric acid	. 000	. 000	. 000	....	....
Caproic acid	. 194	. 000	. 456	....	....
Acids from alcohols	. 769	. 621	. 802	....	....
Acids from esters	. 850	. 840	. 640	. 210	. 200
Succinic acid	. 000	. 000	. 000	....	....
Total lactic acid	Trace.	. 000	. 000	....	....
Racemic lactic acid	....	....	....	....	....
Active lactic acid	....	....	....	....	....

Streptococcus b_1 decomposed all the formic acid present in the milk and produced large quantities of acetic and a little propionic acid. Esters were produced in small amounts. No lactic acid was found.

The cultures of Streptococcus b_2 (Table IV) were 11 weeks old when analyzed. In both flasks a soft curd was deposited. The contents of flask 1 had a sharp nutty odor and flavor. Flask 2 had a sharp, acid, unpleasant taste and a sharp, rancid smell suggesting butyric acid. The cheese from which the isolation was made was 101 days old when examined. It contained this organism in numbers of 1,000,000,000 per gram. The cheese possessed a good Cheddar flavor when 2 weeks old. Later, a sharpness developed, but the cheese remained good for 6 months.

TABLE IV.—*Substances formed by the action of Streptococcus b_2*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids	49.138	55.152	12.165	36.973	42.987
Formic acid	3.640	6.333	5.548	−1.908	.789
Acetic acid	38.370	41.059	6.160	32.210	34.899
Propionic acid	4.903	5.138	.000	4.903	5.138
Butyric acid	.558	.563	.000	.558	.563
Caproic acid	1.667	2.059	.456	1.211	1.603
Acids from alcohols	8.607	3.552	.802	7.805	2.750
Acetic acid	8.209	3.187	....	7.407	2.385
Propionic acid	.398	.365	....	.398	.365
Acids from esters	.250	1.930	.640	....	1.290
Total lactic acid	.000	.000	.000	.000	.000
Racemic lactic acid	....	....	....	....	....
Active lactic acid	....	....	....	....	....

This form of coccus decomposed a part of the formic acid originally present in the medium. The increase in acidity was mainly due to acetic acid, but some propionic and a little caproic acid were also formed. The interesting point in connection with this organism, however, was the strong production of alcohols, amounting to a quantity equivalent to nearly 8 cubic centimeters of decinormal acid. Most of this alcohol was ethyl, a little propyl alcohol making up the remainder. In one flask a marked production of esters was also noted. No lactic acid was produced.

The cultures of Streptococcus b_3 (Table V) were 4½ months old when analyzed. Flask 1 had a yellowish colored solution over a firmly deposited custard-like curd. The solution was acid to litmus and had a pleasant, slightly acid smell. The residue in flask 2 was less than that in flask 1 and was covered by a brown-colored solution which was acid to litmus. Its odor was similar to that of flask 1, but was more pronounced, giving a suggestion of cheese odor.

TABLE V.—*Substances formed by the action of Streptococcus b_3*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids	88. 799	90. 474	14. 530	74. 269	75. 944
Formic acid	3. 153	. 000	8. 295	−5. 142	− 8. 295
Acetic acid	68. 190	69. 150	6. 135	62. 055	63. 015
Propionic acid	10. 209	9. 970	. 000	10. 209	9. 970
Butyric acid	2. 090	3. 821	. 000	2. 090	3. 821
Caproic acid	5. 157	7. 533	. 100	5. 057	7. 433
Acids from alcohol	7. 743	5. 158	. 375	7. 368	4. 783
Formic acid	. 313	. 000	....	. 313	....
Acetic acid	6. 908	4. 612	....	6. 533	....
Propionic acid	. 522	. 546	....	. 522	. 546
Acids from esters	4. 739	4. 856	. 833	3. 906	4. 023
Formic acid	. 443	. 000	....	. 443	. 000
Acetic acid	3. 979	4. 651	....	3. 146	3. 818
Propionic acid	. 317	. 205	....	. 317	. 205
Citric acid	65. 982	47. 090	84. 800	−18. 818	−37. 710
Total lactic acid	. 000	. 000	. 000	. 000	. 000
Racemic lactic acid	....	....	....	....	....
Active lactic acid	....	....	....	....	....
Ammonia .... grams	. 045	. 052	. 023	. 022	. 029

The data in Table V show the same general indications as the data in Table IV. A larger increase was shown in total volatile acidity. Comparatively a much greater increase was noted in the case of butyric and caproic acids. A greater increase was also evident in alcohol and ester production, ethyl alcohol and acetic acid in ester combination predominating. A minute quantity of formic acid previously existing in ester compounds and also from methyl alcohol was recovered. Since probably only about 6.2 per cent of esters are recovered in the method used, the amount of esters actually found indicates a preexisting quantity of those bodies equivalent to 64.8 c. c. N/10. This quantity is greater than the ester content of any cheese examined. If this organism is an agent which produced esters in cheese, as the data indicate, it would, however, be subjected to inhibiting influences in the cheese mass and probably not be able to form esters in such great quantities as when in pure culture.

The culture of Streptococcus d_1 (Table VI) was 2½ months old when analyzed. The medium had a pleasant nutty odor and a slightly acid taste. A soft custard-like curd had formed; that in flask 2 showed the greater bacterial action, having a more acid odor, but the taste was similar to that in flask 1. The curds were alike, with a clear supernatant liquid. The cheese from which this organism was isolated was 133 days old and contained the organism to the extent of 170,000,000 per gram. It had a mild Cheddar flavor after 4 months of curing.

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TABLE VI.—*Substances formed by the action of Streptococcus d_1*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids	19. 142	Lost.	14. 530	4. 612	Lost.
Formic acid	. 000		8. 295	−8. 295	
Acetic acid	18. 310		6. 135	12. 175	
Propionic acid	. 692		. 000	. 692	
Butyric acid	. 000		. 000		
Caproic acid	. 140		. 100		
Acids from alcohol	. 680	1. 000	. 375		0. 625
Acids from esters	. 915	. 725	. 833	. 082	
Citric acid			84. 800		
Total lactic acid		Trace.	. 000		
Racemic lactic acid					
Active lactic acid					

All the formic acid was destroyed by this coccus, and but a comparatively small quantity of acetic acid formed. The activity of this organism was apparently slight; but slight traces of esters were found in one flask and no lactic acid in either flask. The apparent contradiction that there exists a larger content of acetic acid than total volatile acids is due to the destruction of formic acid.

The cultures of Streptococcus d_2 (Table VII) were 2 months old when analyzed. They possessed a pleasant nutty taste and smell. No digestion was apparent. The contents of flask 2 had a trifle more pronounced flavor and odor than in flask 1, but were of the same quality. The cheese from which the organism was isolated was 75 days old and contained this coccus to the extent of 10,000,000,000 per gram. No typical Cheddar flavor had developed. After 5 months the cheese developed a sharpness in taste, but still was fairly good.

TABLE VII.—*Substances formed by the action of Streptococcus d_2*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids	58. 313	66. 664	14. 530	43. 783	52. 134
Formic acid	. 789	1. 029	8. 295	−7. 506	−7. 266
Acetic acid	53. 140	59. 530	6. 135	47. 005	53. 395
Propionic acid	4. 029	5. 700	. 000	4. 020	5. 700
Butyric acid	. 000	. 301	. 000		
Caproic acid	. 355	. 104	. 100		
Acid from alcohols	. 800	. 765	. 375	. 425	. 390
Acid from esters	. 770	. 540	. 833		
Citric acid	7. 100	7. 100	84. 800	−77. 700	−77. 700
Total lactic acid	. 000	. 000	. 000		
Racemic lactic acid					
Active lactic acid					

This culture produced acetic acid almost entirely. As practically all of the citric acid had been destroyed, it may be assumed that this acid was in part the source of the acetic acid. That citric acid can be broken down by certain organisms has already been pointed out by Bosworth and Prucha (1910).

The cultures of Micrococcus b (Table VIII) were 3¼ months old when analyzed. The flasks were alike in appearance and odor. The cheese was 43 days old and had a mild Cheddar flavor when the isolation was made. This organism was present in the cheese to the extent of 1,600,-000 per gram.

TABLE VIII.—*Substances formed by the action of Micrococcus b*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids	72. 785	67. 529	12. 164	60. 621	55. 365
Formic acid	4. 857	3. 667	5. 548	—. 621	—1. 881
Acetic acid	64. 305	59. 740	6. 160	58. 145	53. 580
Propionic acid	3. 220	3. 914	. 000	3. 220	3. 914
Butyric acid	. 000	. 000	. 000	. 000	. 000
Caproic acid	. 403	. 208	. 456		
Acids from alcohol	. 409	Lost.	. 802		
Acids from esters	. 500	. 405	. 640		
Succinic acid	. 000	. 000	. 000	. 000	. 000
Total lactic acid	17. 152	. 000	. 000	17. 152	. 000
Racemic lactic acid	3. 730	. 000	. 000	3. 730	. 000
Active lactic acid	13. 422	. 000	. 000	13. 422	. 000

Acetic acid shows the only large increase among the volatile acids. In flask 1 a small quantity of lactic acid had developed. Most of it was of the active variety.

The cultures of Micrococcus d (Table IX) were 1¾ months old when analyzed. Flask 2 showed from its appearance and odor further decomposition and probably more rapid growth of the organism than flask 1.

TABLE IX.—*Substances formed by the action of Micrococcus d*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids	29. 482	26. 614	12. 164	17. 318	14. 450
Formic acid	2. 484	1. 316	5. 548	—3. 064	—4. 232
Acetic acid	8. 370	13. 295	6. 160	2. 210	7. 135
Propionic acid	4. 540	4. 988	. 000	4. 540	4. 988
Butyric acid	7. 811	2. 456	. 000	7. 811	2. 456
Caproic acid	6. 277	4. 559	. 456	5. 821	4. 103
Acids from alcohol	1. 499	1. 752	. 802	. 697	. 950
Acids from esters	. 350	. 450	. 640		
Succinic acid	. 830	3. 300	. 000	. 830	3. 300
Total lactic acid	3. 396	20. 856	. 000	3. 396	20. 856
Racemic lactic acid	. 000	11. 940	. 000	. 000	11. 940
Active lactic acid	3. 396	8. 916	. 000	3. 396	8. 916

It will be noticed that formic acid has decreased. This will be found true for all the organisms studied, the acid probably being decomposed by the organisms themselves. In flask 1 the greatest increase is shown in the butyric-acid content. In flask 2, where greater decomposition and probably more rapid growth of the organisms occurred, the butyric acid is very much less, while the acetic acid has increased. This would indicate a decomposition of butyric acid to a lower acid, as Duclaux suggests in his theory of the formation of acids lower than butyric. All of the volatile acids, except formic, show an increase. A very small quantity of succinic acid was formed, but no esters and very little alcohol were produced. In flask 2 there was quite an amount of lactic acid, of which most was racemic.

The substances formed by a third Micrococcus are given in Table X. This culture was not classified as to variety. It was isolated from a cheese when the latter was 44 days old and when the organism was present in numbers amounting to 100,000,000 per gram. After 5 months this cheese developed a good Cheddar flavor. In both flasks the media were light brown in color and had a pleasant nutty odor and taste; they showed slight digestion and were but slightly acid to litmus.

TABLE X.—*Substances formed by the action of an unidentified Micrococcus*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids	46.058	52.669	14.530	31.538	38.139
Formic acid	.000	.000	8.295	−8.295	−8.295
Acetic acid	41.850	37.060	6.135	35.715	30.925
Propionic acid	3.999	14.886	.000	3.999	14.886
Butyric acid	.138	.289	.000	.138	.289
Caproic acid	.081	.434	.100		
Acids from alcohols	.990	.700	.375	.525	.325
Acids from esters	1.000	.540	.833	.167	
Citric acid	80.900	59.240	84.800		−25.560
Total lactic acid	.000	.000	.000	.000	.000
Racemic lactic acid					
Active lactic acid					

This organism produced quite a quantity of acetic acid and more propionic acid than any other organism examined. No lactic acid was found.

In order to determine the influence of the presence of alkali on the character of the products formed, a flask of milk to which was added calcium carbonate was inoculated with one of the micrococci. The substances formed were acetic, propionic, butyric, and caproic acids, but no formic acid. The proportion of these acids was very similar to that of the acid formed by Micrococcus d and would indicate that the alkali exerted no influence on the character of the substances formed.

From a summary of all the foregoing data it appears that the coccus forms do not produce formic acid, and, with the exception of Micrococcus b and d, do not produce lactic acid. In the case of these two strains the form of acid produced was both active and racemic. With the exception of Micrococcus d all produce relatively large amounts of acetic acid. Streptococcus b_8 produced a fairly large quantity of butyric and caproic acids.

SUBSTANCES FORMED BY ORGANISMS OF THE BACTERIUM CASEI GROUP

In Tables XI and XII are given data showing the substances formed by the action of a high-acid-producing organism, one of the *Bacterium casei* group. Duplicate flasks 58_1 and 58_2 were prepared from two strains of the same culture obtained from different colonies on an agar plate. The milk media at 7½ months old were light yellow in color, and the curd had settled in a firm mass. Both flasks of 58_1 and flask 1 of 58_2 had a very faint acid odor. Flask 2 of 58_2 had a ripened-cream odor.

TABLE XI.—*Substances formed by the action of culture 58_1*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids	35.128	38.063	14.530	20.598	23.533
Formic acid	.000	1.387	6.295	−6.295	−4.908
Acetic acid	33.420	35.760	6.138	27.285	29.625
Propionic acid	1.708	.916	.000	1.708	0.916
Butyric acid	.000	.000	.000	....	....
Caproic acid	.000	.000	.100	....	....
Acids from alcohol	.250	Lost.	.375	....	....
Acids from esters	.800	Lost.	.833	....	....
Citric acid	.000	.000	84.800	−84.800	−84.800
Total lactic acid	92.648	99.100	.000	92.648	99.100
Racemic lactic acid	44.552	87.800	....	44.552	87.800
Active lactic acid	48.096	11.300	....	48.096	11.300

TABLE XII.—*Substances formed by the action of culture 58_2*

[Computed in cubic centimeters of tenth-normal solution]

Substance.	Quantity found.		Control.	Quantity produced.	
	Flask 1.	Flask 2.		Flask 1.	Flask 2.
Total volatile acids	41.675	40.928	14.530	27.145	26.498
Formic acid	3.408	.984	8.295	−4.887	−7.311
Acetic acid	36.130	31.990	6.135	29.995	25.855
Propionic acid	2.137	7.954	.000	2.137	7.954
Butyric acid	.000	.000	.000	....	....
Caproic acid	.000	.000	.100	....	....
Acids from alcohol	.500	.950	.375	....	....
Acids from esters	1.500	1.000	.833	.667	.167
Citric acid	.000	.000	84.800	−84.800	−84.800
Total lactic acid	48.540	39.970	.000	38.540	39.970
Racemic lactic acid	41.100	20.170	....	41.100	20.170
Active lactic acid	7.440	19.800	....	7.440	19.800

This organism, as might be expected, shows a marked difference from the coccus group in the character of the substances formed. A large amount of lactic acid, including both the racemic and the active forms, was produced. All the citric acid of the milk was destroyed. Like the coccus forms, this organism also produced much acetic acid, but no formic, butyric, or caproic acid. Culture 58_2 produced some esters.

ESTER FORMATION IN CHEDDAR CHEESE

It has been determined that esters do not appear in Cheddar cheese until it is about 5 weeks old. Streptococcus b_3 (see Table V) produced an ester content in the medium equivalent to 64.8 c. c. N/10. To throw some light on the question whether esters could be formed in the cheese or medium from mere mass action of free alcohol and acid, a trial was made with a mixture of these two substances. It is known that the contact of acetic acid and ethyl alcohol can produce esters even without adding a dehydrating agent. Dilute solutions of pure acid and pure alcohol were mixed and allowed to stand for a few months, and then a very slight excess of KOH solution was added. The alcohol and esters were next distilled off. The distillate was saponified with KOH, acidified with H_2SO_4, and distilled repeatedly to obtain the acids which had entered into the ester combination. Blank determinations were carried out to check the purity of all chemicals used. The results are given in Table XIII.

Table XIII.—*Production of ester from the contact of acid and alcohol*

Ethyl alcohol.	Acetic acid.	Result.
Per cent.	*Per cent.*	
1	1	No ester detected.
2	2	Small amount of ester.
5	5	Esters formed.
8	8	Do.
14	14	Do.

Table XIII shows that free acetic acid and alcohol can not form esters in dilute aqueous solutions. Comparing this concentration with that found in cheese, it is probable that the solution of alcohol in the cheese moisture is very dilute—much less than 1 per cent. The greater part of the acids is also combined with basic substances. If these assumptions are accepted, then it can be said that the esters in cheese are probably not produced by mere contact of alcohol and acid but by the intervention of biological activities.

Of course, the question of actual concentration of alcohol or acid in any phase in the cheese mass is not possible of definite statement. There may be very little "free" water in the cheese, most of it being in com-

bination with the cheese colloids; consequently the concentration of acid or alcohol in such water may be very large, thereby affording an opportunity for ester formation by mass action. On the other hand, it must not be assumed that the alcohols or acids are "free" in such a complex system, but may also be in combination with the colloids of the cheese mass. If this last alternative is permissible—and the writers believe it to be true, especially for the acids—then there is some reason, at least, for the assumption that ester formation is not the result of mere contact of acid and alcohol, but occurs through the intervention of some agent which shifts the point of equilibrium in the system toward ester stability.

To determine whether inherent milk enzyms acting in curing cheese could produce esters, alcohols, or volatile acids, a cheese was made from chloroformed milk and kept in an atmosphere of chloroform for 5 months. To determine the volatile bodies, 800 grams of the cheese were submitted to steam distillation, after acidifying with H_2SO_4. The entire analytical process was conducted as has been described. The results were negative, there being neither acids nor esters. This shows that inherent milk enzyms are not the cause of the production of volatile fatty acids and esters in curing cheese. From this experiment it is apparent that the inherent lipase in milk is either retarded in its action by chloroform or else is very slow in its action.

AMMONIA PRODUCTION IN MILK

The origin of ammonia in ripening cheese had been ascribed by Babcock and Russell (1897, p. 161) and Babcock, Russell, Vivian, and Hastings (1899, p. 157) to the action of galactase. In further work on this problem Van Slyke and Hart (1903) showed that in chloroformed cheese, where galactase and pepsin would be the only proteolytic agents present, no ammonia was formed. To throw further light on this problem, cultures of a few of the organisms known to be active in Cheddar cheese were examined for ammonia production. Milk was the medium used for these determinations. A part of this medium, in the case of the coccus group, was distilled directly with MgO. In the case of *Bacterium casei*, the medium was first treated with tannic acid and salt solution according to the standard methods for separation of the tannin precipitate, and the ammonia determined in the filtrate by distillation with MgO. In Table XIV are recorded the results.

TABLE XIV.—*Quantity of ammonia produced in 300 c. c. of milk by different organisms*

Organism.	Quantity of NH_3 found.	Quantity of NH_3 in control.	Quantity of NH_3 formed by organisms.
	Grams.	*Grams.*	*Grams.*
Streptococcus b_2	0.0220	0.023	......
	.0200	.023	....
Streptococcus b_3	.0450	.023	0.0220
	.0520	.023	.0290
Micrococcus	.0690	.023	.0460
Bacterium casei$_1$	.0427	.026	.0167
	.0382	.026	.0122
Bacterium casei$_2$	.0367	.026	.0107
	.0453	.026	.0193

No large quantity of ammonia was formed by any of the organisms examined. The difference in the amount of ammonia produced by Streptococcus b_2 and Streptococcus b_3—two strains of the same variety—may be due to the fact that Streptococcus b_3 grew for more than twice as long a time as did the other. It is clear from Table XIV that some of the biological agencies active in the cheese are capable of forming both acids and ammonia.

KINDS OF LACTIC ACID IN CHEESE[1]

In considering lactic acid and its changes in cheese, it will be remembered that lactose disappears from the cheese mass after a very few days of curing; subsequently the lactic acid increases up to the five weeks' stage. At later periods the lactic-acid content fluctuates, probably the result of production and decomposition by active organisms. Thus, there seems to be a source of lactic acid other than lactose. A solution of alanin, one of the amino acids arising from casein proteolysis and very closely related to lactic acid, was inoculated with a piece of old cheese, in order to ascertain whether alanin could be a source of lactic acid (Suzuki, Hastings, and Hart, 1910). The results were negative, but it is possible that either the nature of the solution or the age of the cheese was responsible for this result. Additional work on this point is necessary.

It is known that cheese contains lactic acid, which usually is racemic in variety. It has been shown in the preceding article that Cheddar cheese 4 or 5 days old contains both racemic and active lactic acid, the latter being present in much greater amount than the former. The active form gradually decreases until it disappears, while the racemic acid increases and remains. It was found by Salkowski (1909, p. 237) that the transformation of dextro lactic acid into racemic acid on pro-

[1] The work reported in the remainder of this paper was completed before the classification of cheese organisms referred to on page 195 and treated in detail in the preceding article entitled "Bacteria concerned in the production of the characteristic flavor of cheese of the Cheddar type" was adopted; consequently that classification is ignored in the following pages.

longed standing takes place in a meat extract such as Liebig's. In the curing of cheese the disappearance of active lactic acid, as well as the production of racemic lactic acid, takes place rapidly. The early stages of these phenomena were next investigated.

Whey drawn from the vat during the process of cheese making and subjected to analysis for lactic acid gave the results shown in Table XV:

TABLE XV.—*Analysis of whey, showing quantity of lactic acid as zinc lactate*

Fraction No.	Crystals of zinc lactate.	Water of crystallization.[1]
	Grams.	*Per cent.*
1	2.4587	13.07
2	.7902	12.27

[1] The theoretical percentage for water of crystallization in active zinc lactate is 12.89.

Fresh curd from which the above whey was drawn was kept at 35° C. for 3 days and gave the following results (Table XVI):

TABLE XVI.—*Analysis of fresh curd, 3 days old, showing quantity of lactic acid as zinc lactate*

Fraction No.	Crystals of zinc lactate.	Water of crystallization.[1]
	Grams.	*Per cent.*
1	2.0837	17.99
2	.1652	17.43

[1] The theoretical percentage for water of crystallization in racemic zinc lactate is 18.18.

It is seen that whey contained active lactic acid, while curd or cheese only 3 days old and kept at 35° C. contained nearly all its lactic acid in the racemic form. It is probable that a second group of organisms follows the early action of the predominating active lactic-acid producers in the cheese during the first 3 days. There is also a possibility that a somewhat different sequence of bacterial life occurs in the whey from that which takes place in the curd, with the result that active acid is produced in whey and the racemic variety in the curd. To settle this point, whey and curd were investigated for the forms of lactic acid occurring in them. The results are given in Table XVII:

TABLE XVII.—*Analysis of whey and curd, showing the quantity of lactic acid as zinc lactate*

Fraction No.	Whey when drawn.		Curd at hooping time.	
	Crystals of zinc lactate.	Water of crystallization.	Crystals of zinc lactate.	Water of crystallization.
	Grams.	*Per cent.*	*Grams.*	*Per cent.*
1	0.4570	13.65	0.1974	12.36
2	1.6520	12.50	....	....

Table XVII shows that the whey and curd contained active lactic acid and had a similar course of fermentation during the very early stages. A different fermentation evidently took place in the cheese after pressing, but not in the curd stage. The next question that arose was, At what stage in the curing process did the production of racemic lactic acid take place? The data in Table XVIII show that racemic acid begins to appear very soon after going to press.

Table XVIII.—*Analysis of whey, curd, and cheese, showing the quantity of lactic acid as zinc lactate*

Fraction No.	Whey when drawn.		Curd at hooping time.		Whey kept at temperature of curd and stood overnight in pressing room.		Cheese 24 hours old.		Cheese 48 hours old.	
	Crystals of zinc lactate.	Water of crystallization.	Crystals of zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.	Crystals of zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.
	Grams.	*Per ct.*	*Grams.*	*Per ct.*	*Grams.*	*Per ct.*	*Grams.*	*Per ct.*	*Grams.*	*Per ct.*
1	0.2360	13.64	0.3044	13.00	0.6435	13.05	0.0533	17.07	0.1123	17.45
2	.0000	....	.0000	....	.8010	13.54	.0846	17.37	.1420	17.11
3	....	....	....	....	.2745	13.47	.1260	17.77	.5805	17.07
4	....	....	....	....	.3863	12.96	.2606	14.19	.0565	14.69
5	....	....	....	....	.2788	13.12	.4948	13.29	.4555	13.63
6	....	....	....	....	.0975	13.53	....	....	....	....

Whey when drawn, and also after standing overnight, contained active lactic acid. Curds at hooping time contained active lactic acid. One-day-old and two-day-old cheese contains a mixture of racemic and active acid.

The causes for the early production of racemic acid and the disappearance of active acid may be ascribed to a direct production by either enzyms or bacterial action of active acid which is of opposite polarity from that already present.

In order to study the relation of enzymic action in curd to this problem, the following experiment was performed: Curd at hooping time was divided into five parts. The first portion was immediately analyzed for lactic acid; the second portion was analyzed after standing 46 hours in the pressing room; the third portion was kept for 17 days at 35° C.; the fourth part was treated with chloroform and kept for 17 days at room temperature; the fifth portion was treated with chloroform and stood for 3 months at room temperature. The data secured on the nature of the lactic acid produced are shown in Table XIX.

TABLE XIX.—*Analysis of curd, showing lactic acid as zinc lactate*

Fraction No.	Curd at hooping time.		Curd kept 46 hours in pressing room.			Curd kept 46 hours at 35° C.		Curd kept 17 days with chloroform.		Curd kept 3 months with chloroform.	
	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.	Zinc oxid in lactate.	Zinc lactate.	Water of crystallization.	Zinc lactate	Water of crystallization.	Zinc lactate.	Water of crystallization.
	Grams.	*P. ct.*	*Grams.*	*P. ct.*	*P. ct.*	*Grams.*	*P. ct.*	*Grams.*	*P. ct.*	*Grams.*	*P. ct.*
1	0.1902	12.92	0.5601	15.97	33.8	0.6515	18.00	0.2637	12.96	0.1490	17.92
2	.0000	....	.5854	14.14	33.9	.6040	18.11	.0000	....	.2183	14.13
3	....	....	.2314	13.48	....	.3901	17.86	....	....	.2119	12.79
4	....	....	.0000	....	....	.0000	....	....	....	1.0895	12.78
5	....	....	....	....	....	....	....	....	....	.7635	....
6	....	....	....	....	....	....	....	....	....	.2074	13.21
7	....	....	....	....	....	....	....	....	....	.1546	13.06

In order to verify the results secured on the increase of racemic acid and the decrease of active acid in fresh cheese curd, as shown above, another sample of fresh curd was divided into three portions. One portion was examined immediately for lactic acid, another after 24 hours, and the third portion after keeping at 60° for 48 hours. See Table XX.

TABLE XX.—*Analysis of curd and fresh cheese, showing lactic acid as zinc lactate*

Fraction No.	Fresh curd.			One-day-old cheese.			Two-day-old cheese.		
	Zinc lactate.	Water of crystallization.	Zinc oxide.	Zinc lactate.	Water of crystallization.	Zinc oxid.	Zinc lactate.	Water of crystallization.	Zinc oxid.
	Grams.	*Per cent.*	*Per cent.*	*Grams.*	*Per cent*	*Per cent.*	*Grams.*	*Per cent.*	*Per cent.*
1	0.2704	12.52	33.6	0.1786	17.19	32.7	0.1670	17.25	33.0
2	.0617	11.80	33.4	.0975	12.20	33.3	.0457	17.07	33.2
3	.0000	....	....	.0778	12.90	33.9	.1946	12.90	33.8
4	....	....	....	.1514	12.30	33.8	.2472	13.50	33.9
5	....	....	....	.2133	12.00	33.6	.0551	12.00	34.0
Total.	.3321	....	....	.7186	....	....	.7096	....	....

The theoretical percentage for water of crystallization in racemic zinc lactate is 18.18; in active zinc lactate it is 12.89 per cent. The theoretical percentage of ZnO in anhydrous zinc lactate is 33.3

From Table XX it is clear that in fresh curd, which contains active lactic acid, the production of racemic acid begins after about 24 hours at room temperature and that this production is accelerated by a temperature of 35° C. In the curd kept with chloroform for 17 days the production of racemic acid appeared to have been checked, while curds kept with chloroform for 3 months gave a small amount of the racemic variety. A parallel case was found by Saiki (1909) in the autolysis of a normal dog's liver, even in a strictly sterile solution. In his experiments racemic lactic acid gradually formed. This enzymic action may be considered a partial cause of the appearance of the racemic acid. The question whether enzymic action decomposes the lactose of the curd

into racemic acid or causes a production of an active acid of opposite polarity to the acid already present has not been settled. It has been shown (Hastings, Evans, and Hart) that the *Bacterium lactis acidi* does produce enzyms, and it may be that these enzyms are one of the factors in the production of racemic acid, although it is more probable that, because of the very slowness of the enzymic action, the real factor is an increasing number of active bacteria of different types from the *B. lactis acidi.*

The question whether *B. lactis acidi* or its enzym is the cause of the disappearance of active lactic acid and the appearance of racemic acid must be considered. It is known that lactic acid isolated from a lactose solution inoculated with *B. lactis acidi* is active in variety and not racemic. Even after prolonged standing, the lactic acid is found to be active. For this reason it is not believed that *B. lactis acidi* is the direct cause of this change. To determine whether the enzym of *B. lactis acidi* is the cause of this transformation, a solution containing active lactic acid, formed by inoculation with this organism and after several days treated with toluol, was allowed to stand for 2 months at 35° C. At the end of this time all of the lactic acid isolated was found to be active in variety, as shown by Table XXI.

TABLE XXI.—*Analysis of a solution of toluolated active lactic acid, showing active lactic acid*

Fraction No.	Zinc lactate.	Water of crystallization.
	Grams.	*Per cent.*
1	0.1148	12.98
2	.4812	12.96
3	1.0186	12.99
4	1.3713	13.06

Further, it was thought possible that the kind of lactic acid produced by *B. lactis acidi* might be influenced by temperature conditions. In order to test this, a lactose solution containing 3.6 per cent of lactose, 1 per cent of peptone, and 10 grams of calcium carbonate to 300 c. c. of the solution was inoculated with this organism and put in the ice box. After 38 days the lactic acid isolated was found to be active in form (0.1303 gram of zinc lactate gave 13.04 per cent of water of crystallization); hence it is clear that low temperature does not change the direction of the reaction.

The foregoing experiments lead to the conclusion that the *B. lactis acidi* examined or its enzym, either in the presence or absence of antiseptics, is not the direct cause of the disappearance of active lactic acid and the appearance of racemic acid. Probably the same conclusion is applicable to cheese curd.

ACTION OF OTHER GROUPS OF BACTERIA

Consideration must now be given to organisms other than *Bacterium lactis acidi* as an explanation of the change in optical activity of lactic acid in cheese.

A yellow Micrococcus was isolated from cheese and inoculated into a lactose solution containing 5 per cent of lactose, 1 per cent of peptone, and 10 grams of $CaCO_3$ to 300 c. c. of solution. After 48 hours of incubation, toluol was added. According to analyses made 72, 82, and 105 days after adding the toluol, the quantity of lactose remained constant.

Therefore, the yellow coccus, in this case at least, had no enzymic action on lactose in the presence of toluol. No lactic acid could be isolated from media similar to the above, inoculated with the same coccus, and incubated without antiseptics. This is further corroborative of the fact that the coccus group, as a group, is not a lactic-acid producer and consequently could have no large part in the lactic-acid changes observed in the curds.

In order to ascertain whether the presence of the Micrococcus has some influence on *Bacterium lactis acidi* in the latter's action on milk sugar, a mixture of the two bacteria was inoculated into a lactose solution containing peptone and calcium carbonate. The results show that active acid was produced, but not racemic acid, as 3.45 grams of zinc-lactate crystals were obtained, containing 12.96 per cent of water of crystallization. It should, however, be remembered that in this group were found two strains (Tables VIII and IX) which could produce lactic acid on milk media. From the above experiment, where a Micrococcus was inoculated alone into a lactose solution, no lactic acid was obtained, but when this organism, presumably the same one, was inoculated into milk a quantity of lactic acid was produced. The ether extract from 300 c. c. of milk which had been inoculated with the organism gave over 100 c. c. of N/10 acidity. Upon neutralizing with $Ba(OH)_2$ a voluminous precipitate occurred. The filtrate from this precipitate required about 40 c. c. of N/10 $ZnSO_4$ solution to take up the barium present. The solution of zinc salts was evaporated to a small quantity and allowed to stand in an ice box for crystallization. There was obtained 0.1031 gram of crystals, which contained 17.53 per cent of water of crystallization. As this is very close to the theoretical percentage of water of crystallization in racemic zinc lactate (18.18 per cent), very nearly all of the lactic acid thus formed was racemic. It would be easy to infer that the organisms from the coccus group discussed above were able to produce very different end-products with some variation in the nature of the media. It is, however, more than probable that the organisms dealt with were distinct in type and physiological action and that the second coccus discussed was one of the strains capable of producing lactic acid.

The next organisms investigated with respect to forms of lactic acid produced were those which belong to the *Bacterium casei* group and which produce a much higher acidity than *B. lactis acidi*, although they grow more slowly than the latter. Several solutions of sterile milk and calcium carbonate inoculated with a culture of *B. casei* gave, on incubation, levo-lactic acid, although Heinemann (1909), experimenting in the same direction with members of this group, obtained racemic acid. Another culture which was isolated from milk gave dextro acid instead of the levo form. These were evidently two different varieties of *B. casei*. They will be designated "*Bacterium casei* 1" and "*Bacterium casei* 2."

Bacterium casei 2 was inoculated into 250 c. c. of sterile milk. Calcium carbonate was added, and the medium was allowed to stand for 7½ months. The liquid of the medium had almost evaporated at the end of that time, and crystals had deposited on the bottom of the flask. These crystals were purified by repeated crystallization and then dried in the desiccator. In the resulting product was found 18.07 per cent of calcium, the theoretical percentage of calcium being 18.34 for anhydrous calcium lactate. Rough isolation of lactic acid gave about 0.900 gram of zinc-lactate crystals. Fractional crystallation proved that it was active, the salt being levo-rotatory—that is, the free acid was dextrorotatory.

Pure cultures of *Bacterium lactis acidi* and *B. casei* and mixtures of these two cultures with and without calcium carbonate were examined, to determine the types of lactic acid present.

Lactic acid was isolated. Its zinc salt was fractionally crystallized, and the water of crystallization was estimated in each fraction of crystals. See Table XXII.

TABLE XXII.—*Analysis of 300 c. c. of sterilized milk, showing production of lactic acid by Bacterium lactis acidi and Bacterium casei 1*

Fraction No.	I. Bacterium lactis acidi.		II. Bacterium lactis acidi and B. casei 1.		III. Bacterium lactis acidi and B. casei 1 with calcium carbonate.		IV. Bacterium casei 1.	
	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.
	Grams.	*Per cent.*	*Grams.*	*Per cent.*	*Grams.*	*Per cent.*	*Grams.*	*Per cent.*
1	1.66	....	1.47	18.06	4.40	18.36	2.54	12.87
2	.73	13.36	.92	17.25	3.30	12.75	.16	12.88
3	....	....	.06	17.95	3.00	13.24	.16	12.89
4	....	....	....	....	3.00	12.90	....	....
5	....	....	....	....	.34	12.26	....	....
6	....	....	....	....	.65	11.98	....	....
	Levo-salt.				Dextro-salt.		Dextro-salt.	

Bacterium lactis acidi produced levo zinc lactate, as has already been shown, and *B. casei* 1 gave dextro zinc lactate. The mixture of the two gave racemic acid, as shown in section II. It may be that in curing cheese after pressing, factors similar to those used in these experiments are operative in the production of the racemic variety of lactic acid.

The data in section III, which were obtained from the mixed culture of *Bacterium lactis acidi* and *B. casei* containing calcium carbonate, show racemic zinc salt and also active acid, the latter being produced, no doubt, by *B. casei* 1, the activity of which continues after that of *Bacterium lactis acidi.*

Another experiment, with *Bacterium casei* cultures 1, 2, and 3, the last-named being a pure culture supposedly of a different type from 1 and 2, was carried out as in the previous experiments. The results are given in Table XXIII.

TABLE XXIII.—*Analysis of medium consisting of 200 c. c. of sterile milk and 6 grams of calcium carbonate, showing the production of lactic acid from Bacterium casei 1, 2, and 3*

Fraction No.	Culture 2.		Cultures 1 and 2.		Culture 3.	
	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.	Zinc lactate.	Water of crystallization.
	Grams.	*Per cent.*	*Grams.*	*Per cent.*	*Grams.*	*Per cent.*
1	3.40	12.91	13.42	18.00	2.12	12.94
2	.32	13.03	.71	16.14	4.19	15.12
3	.28	13.00	.64	14.09	.00	....
4	.00	....	.00	......	..	....
	Levo-salt.		Dextro-salt.		Dextro-salt.	

The data show that the mixture of *Bacterium casei* 1 and 2, which in pure cultures produce the two different active lactic acids, gives racemic acid with a slight excess of levo-acid produced by culture 1. This phenomenon might also take place in cheese ripening, producing racemic acid. *Bacterium casei* 3 produces dextro zinc lactate just as culture 1 does, and it also produces the same kinds of volatile fatty acids. Therefore, culture 3 may have been identical with culture 1.

From the foregoing experiments it may at least be concluded tentatively that the formation of racemic lactic acid in Cheddar cheese soon after going to press is due to the later development of organisms of the *Bacterium casei* group principally, together with the possibility that certain forms of the coccus groups can likewise produce racemic lactic acid.

SUMMARY

(1) Representatives of the coccus groups of organisms isolated from Cheddar cheese when grown in milk produced large quantities of the volatile acids, particularly acetic acid. These acids were produced from

citric acid or lactose or protein, as the medium was practically free from fat. These organisms did not produce formic acid. As they are present at times in very large numbers in cheese, they, no doubt, produce much of the volatile fatty acids which arise during the ripening process.

(2) One of the strains of Streptococcus b was found to produce comparatively large quantities of alcohols and esters—bodies which contribute in a large degree to the flavor of cheese.

(3) A dilute solution of acetic acid and alcohol formed esters by mere contact, without bacterial action. In cheese, however, the dilution is probably too great for this manner of ester formation.

(4) Lactic acid was generally not formed by the coccus groups.

(5) The representatives of the *Bacterium casei* group examined gave results differing from those obtained from the coccus forms. They produced no formic acid, but did form some propionic and much acetic acid.

(6) These organisms produced a large quantity of lactic acid, both active and racemic, and decomposed the citric acid of the media.

(7) Cheese made from chloroformed fresh milk did not yield any volatile fatty acids, showing that inherent milk enzyms are not capable of producing these bodies in any appreciable quantity.

(8) Representatives of both the coccus and *Bacterium casei* groups were able to produce ammonia from milk.

(9) Whey and fresh curds contained active lactic acid. Cheese 1 day old contained a mixture of active and racemic lactic acids.

(10) The cause of the disappearance of active lactic acid and the appearance of racemic acid may be due to enzymic action, combined with the action of those bacteria which can produce both kinds of acid.

(11) Some representatives of the *Bacterium casei* group produced levo lactic acid and others dextro lactic acid from milk. A mixture of these two varieties produced racemic lactic acid. A mixture of *B. lactis acidi* and a levo-producing member of the *B. casei* group gave racemic and active lactic acid. The active acid was probably the result of the longer continued activity of *B. casei.*

(12) Racemic lactic acid found in curing cheese may therefore be produced in a small degree by enzym action, but more probably by the combined action of *Bacterium lactis acidi* and the organisms of the *B. casei* group.

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